ORIGINAL ARTICLES

Exhibit C

Serum and Urinary Concentrations of Type IV Collagen and Laminin as a Marker of Microangiopathy in Diabetes

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Serum and urinary concentrations of type IV collagen and laminin were measured by enzyme-linked immunosorbent assay (ELISA) in diabetic patients and compared with normal control subjects. In diabetic patients with proteinuria or with renal insufficiency, serum and urinary concentrations of type IV collagen were higher than those of control subjects ($p < 0.005$). Furthermore urinary concentrations of type IV collagen and laminin were significantly higher in diabetes, even in the absence of nephropathy, than in normal controls ($p < 0.05$). Urinary concentrations of type IV collagen in patients with diabetes and microalbuminuria (0.73 ± 0.11 mg $mmol^{-1}$) were significantly higher than in diabetic patients without nephropathy (0.40 ± 0.060 mg $mmol^{-1}$) ($p < 0.025$). Urinary concentrations of type IV collagen may have a role as an indicator of early diabetic nephropathy. Serum concentrations of type IV collagen in diabetic patients with retinopathy were significantly higher than in normal controls ($p < 0.025$). However, urinary concentrations of type IV collagen ($p < 0.05$) and serum concentrations of laminin ($p < 0.025$) were significantly higher in diabetic patients than normal controls and the difference between patients with and without retinopathy was not significant.

KEY WORDS Basement membrane Type IV collagen Laminin Microangiopathy Type 2 diabetes mellitus

Introduction

Morphological, biochemical, and functional alterations in capillary basement membranes of various organs and tissues in patients with diabetes have been reported. Biochemical and immunohistochemical approaches have been used to characterize the changes of basement membrane components, especially those of the glomerular basement membrane, which include the collagenous component type IV collagen and non-collagenous components laminin, heparan sulphate proteoglycan, and fibronectin.

Recently, radioimmunochemical methods have become available for the study of basement membrane metabolism through measurement of serum and urinary concentrations of basement membrane proteins such as type IV collagen and laminin or their fragments.[1–11] Earlier studies have demonstrated increased serum levels of 7S collagen which is C-terminal domain of type IV collagen in rats with streptozotocin-induced diabetes, levels which can be normalized by insulin treatment.[4] Serum levels of 7S collagen and laminin P1 in patients with diabetes were also found to be increased in comparison to those in the control groups.[2] Serum concentrations of laminin were increased in advanced diabetic nephropathy, and diabetic retinopathy was not found to influence serum concentration of laminin.[7] In the present study, our aim was to measure both serum and urinary concentrations of type IV collagen and laminin by means of enzyme-linked immunosorbent assay (ELISA) in diabetic patients. The intention was to apply these methods to the monitoring of possible changes in basement membrane metabolism in diabetes and to investigate whether serum and urinary concentrations of type IV collagen and laminin might be a useful indicator of microangiopathic complications in diabetes.

Patients and Methods

Patients

We studied 73 patients (29 males, 44 females) with type 2 (non-insulin dependent) diabetes mellitus. Serum and urine from 36 healthy subjects were used as controls. Clinical characteristics of the people studied are shown in Table 1. Patients with additional diseases associated with connective tissue alterations such as rheumatic disorders or chronic liver disease were excluded from the study.

Methods

Type IV collagen was measured with a sandwich enzyme immunoassay (EIA) kit with two monoclonal antibodies to the 7S domain and some of the non-7S and non-NC1 domains (IV-EIA; Fuji Chemical, Takaoka City,

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Table 1. Clinical characteristics of the control subjects and diabetic patients studied

	Normal	No nephropathy	Microalbuminuria	Proteinuria	Renal impairment
n	36	18	24	19	12
Age (years)	48.0 (3.0)	51.2 (2.7)	54.7 (2.5)	54 (1.7)	56 (1.7)
Sex (male/female)	16/20	7/11	9/15	9/10	4/8
Duration of diabetes (years)	–	8.1 (1.7)	7.4 (1.2)	11.1 (1.4)*	17.4 (2.9)**
Hypertension (presence/absence)	–	5/13	6/18	8/10	8/4
HbA_{1c} (%)	–	7.3 (0.25)	7.6 (0.42)	7.8 (0.37)	7.7 (0.30)
Treatment of diabetes mellitus:					
diet alone		8	8	2	1
with oral hypoglycaemic drugs		6	10	8	5
with insulin		4	6	9	6

Mean (SE), or number.
* $p < 0.05$ and ** $p < 0.005$ compared to diabetic patients without nephropathy (DM1)
HbA_{1c} normal reference range 4.3–6.2 %

Japan.)[5,6,9] Briefly, each well of a microlitre plate was coated with 100 µl of monoclonal antibody solution (100 µg IgC ml^{-1}) at 4 °C overnight. This antibody recognizes the NH_2-terminal 7S domain of type IV collagen molecule. In another uncoated plate, 50 µl of serum samples or standard antigen solution was incubated with 200 µl of another monoclonal antibody solution (80 ng peroxidase-labelled Fab μl^{-1} 10^{-2}). This antibody recognizes the central triple-helical domain near the COOH-terminal of type IV collagen molecule. Subsequently, 100 µl of the sample mixture was transferred to the washed antibody-coated plate and incubated for 1 h at room temperature. After washing the plate, 100 µl of the enzyme substrate containing 4 g l^{-1} o-phenylene-diamine and 0.2 ml l^{-1} hydrogen peroxide was added and incubated for 15 min at room temperature. The enzyme reaction was stopped by adding 100 µl 1.33 mol l^{-1} sulphuric acid. The absorbance of the solution was measured at 500 nm to calculate serum concentration of type IV collagen.

Laminin was measured with a sandwich EIA kit (Fujl Chemical) for human serum immunoreactive laminin using a pair of monoclonal antibodies prepared against human placental laminin P1 fragment. The assay was characterized by carrying out two immunoreactions simultaneously, laminin P1 fragment which reacted with both a monoclonal antibody as a solid phase and a horseradish peroxidase labelled monoclonal antibody (Fab') against human laminin P1 fragment as conjugate. A 20 µl portion of laminin P1 fragments as standard solution or serum was mixed with 100 µl mouse monoclonal antibody, Fab'-POD conjugate (1 mg l^{-1}) in a 30 mmol l^{-1} Na phosphate buffer in the Falcon 86 wells-flexible assay plate. A 100 µl portion of the solution was then added to each of the wells coated with monoclonal antibodies; the microplate was left for 60 min at room temperature without shaking.

After washing the microplate twice with saline, the POD activity bound to the microplate was assayed for 15 min at room temperature in a solution (100 µl) of 0.1 mol l^{-1} citric acid/0.2 mol l^{-1} Na phosphate buffer, pH 5.0, containing 46 mmol l^{-1} o-phenylene-diamine and 0.02 ml l^{-1} hydrogen peroxide. The reaction was stopped by adding 100 µl sulphuric acid (1 mol l^{-1}) and the absorbance at 492 nm was measured in a microplate reader (Toso Model MPR-A4, Tokyo, Japan). The concentration of laminin was calculated from the standard curve.

Proteinuria was checked by test paper (Multisticks SG-L, Miles-Sankyo, Tokyo, Japan). For the patients without proteinuria or with intermittent urinary protein excretion, the early change of diabetic nephropathy was examined by urinary albumin: creatinine ratio. Urinary albumin concentration was measured by radioimmunoassay and urinary albumin:creatinine ratio was calculated from urinary albumin concentration divided by urinary creatinine concentration. The degree of diabetic nephropathy was rated as 1 to 4 for each patient:

1. Microalbumin negative group: urinary albumin:creatinine ratio ≤ 60 mg $mmol^{-1}$.
2. Microalbuminuria group: no proteinuria but albumin:creatinine ratio > 60 mg $mmol^{-1}$.
3. Proteinuria group: proteinuria but serum creatinine levels < 177 µmol l^{-1}.
4. Chronic renal failure group: serum creatinine levels ≥ 177 µmol l^{-1} (306 ± 53.6 µmol l^{-1} (mean ± SE)).

Urine was filtered by filter paper, then concentrated 20 times by dialysis against polyethyleneglycol. Urinary concentrations of type IV collagen were divided by urinary concentrations of creatinine to exclude influence of urine volume.

Glycosylated haemoglobin (HbA_{1c}) was measured by a high-performance liquid chromatography.

Retinopathy was determined by ophthalmoscopy and classified as without retinopathy or with retinopathy including simple, preproliferative, and proliferative diabetic retinopathy.

Statistical Analysis

Mean value, standard deviation, and SE were calculated, and statistical analysis (Wilcoxon's test) was performed to test for significance of the differences between the values for each experimental group and those of the control group. Spearman's rank correlation test was used for the calculation of the coefficient of correlation.

Results

There was an overall correlation between concentrations of type IV collagen and laminin in sera ($r = 0.35$, $p < 0.01$) (Figure 1) and in urine ($r = 0.81$, $p < 0.001$).

In patients with diabetes there was no correlation between HbA_{1c} level and serum or urinary type IV collagen or laminin.

In patients with diabetes, significantly higher serum concentrations of type IV collagen were found in the group with proteinuria or raised creatinine than in normal controls (Table 2). Urinary concentrations of type IV collagen were significantly higher in diabetic patients, even those without nephropathy, than in normal controls (Table 2). Furthermore urinary concentrations of type IV collagen in patients with microalbuminuria were significantly higher in those without nephropathy. There was a correlation between urinary concentrations of type IV collagen and albumin:creatinine ratio in these groups ($r = 0.47$, $p < 0.01$). However, there was no significant correlation between urinary concentrations of laminin and albumin:creatinine ratio in these groups.

Table 2. Serum and urinary concentrations of type IV collagen in the diabetic patients and control subjects

	Serum type IV collagen $\mu g\ l^{-1}$	(n)	Urinary type IV collagen mg $mmol^{-1}$	(n)
Normal	63.7 (3.5)	(18)	0.28 (0.030)	(36)
No nephropathy	70.6 (4.9)	(18)	[c]0.40 (0.060)	(17)[a]
Microalbuminuria	70.9 (4.5)	(23)	0.73 (0.11)	(24)[b]
Proteinuria	123.3 (21)	(19)	1.12 (0.23)	(13)[b]
Renal impairment	144.1 (16)	(12)[b]	2.45 (1.03)	(4)[b]

Mean (SE), or number.
[a] $p < 0.05$ and [b] $p < 0.005$ compared to control subjects.
[c] $p < 0.025$ vs all other diabetic groups.

Serum concentrations of laminin were significantly higher in patients with renal impairment than in normal controls (Table 3). Urinary concentrations of laminin were significantly higher in diabetic patients, even those without nephropathy, than in normal controls (Table 3). However, the difference between diabetic patients without nephropathy and with microalbuminuria was not significant.

Serum concentrations of type IV collagen in diabetic patients with retinopathy were significantly higher than in normal controls (Table 4). However, urinary concentrations of type IV collagen and serum concentrations of laminin were significantly higher in all the patients with

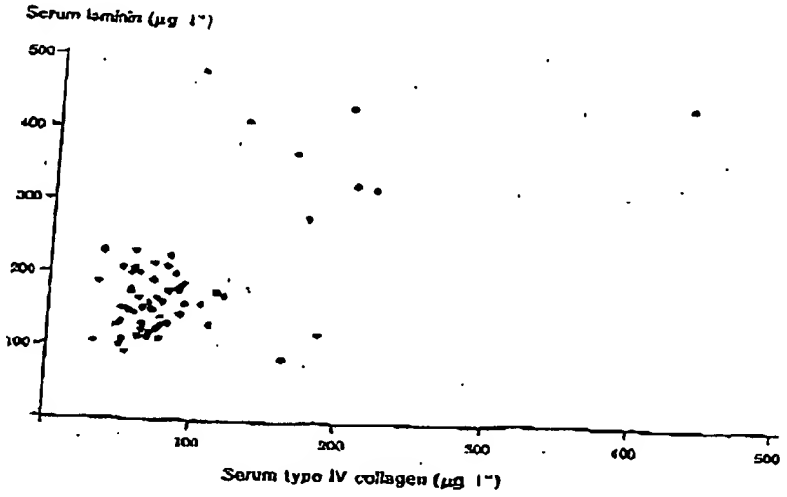

Figure 1. Correlation between concentrations of type IV collagen and laminin in sera; $r = 0.35$, $p < 0.01$

Table 3. Serum and urinary concentrations of laminin in the diabetic patients and control subjects

	Serum laminin μg l⁻¹	(n)	Urinary laminin mg mmol⁻¹	(n)
Normal	144 (10.5)	(14)	0.61 (0.048)	(36)
No nephropathy	144 (9.0)	(9)	0.85 (0.11)	(17)[a]
Microalbuminuria	160 (6.5)	(19)	1.21 (0.15)	(23)[b]
Proteinuria	208 (29.1)	(15)	1.66 (0.54)	(14)[b]
Renal impairment	321 (40.5)	(8)[b]	1.82 (0.39)	(5)[b]

Mean (SD), or number.
[a] $p \leq 0.05$ and [b] $p < 0.005$ compared to control subjects.

Table 4. Serum and urinary concentrations of type IV collagen in the diabetic patients with (+) and without (−) retinopathy and control subjects

	Serum type IV collagen μg l⁻¹	(n)	Urinary type IV collagen mg mmol⁻¹	(n)
Normal	63.7 (3.5)	(18)	0.28 (0.030)	(36)
Retinopathy (−)	88.8 (22)	(17)	0.63 (0.11)	(18)[b]
without nephropathy	66.3 (8.6)	(4)	0.28 (0.079)	(7)
with nephropathy	95.7 (29)	(13)	0.85 (0.14)	(11)[a]
Retinopathy (+)	99.4 (20)	(8)[a]	1.34 (0.50)	(11)[a]
without nephropathy	81	(1)	0.46 (0.22)	(2)
with nephropathy	102 (22)	(7)[a]	1.54 (0.53)	(9)[a]

Mean (SE), or number.
[a] $p < 0.025$ and [b] $p < 0.05$ compared to control subjects.

Table 5. Serum and urinary concentrations of laminin in the diabetic patients with (+) and without (−) retinopathy and control subjects

	Serum laminin μg l⁻¹		(n)	Urinary laminin mg mmol⁻¹	(n)
Normal	144	(10.5)	(14)	0.61 (0.048)	(36)
Retinopathy (−)	194	(24.7)	(12)[a]	0.91 (0.16)	(17)
without nephropathy	159.5	(8.5)	(2)	0.53 (0.12)	(7)
with nephropathy	201	(29)	(10)[a]	1.18 (0.23)	(10)[a]
Retinopathy (+)	251	(48.9)	(8)[a]	1.60 (0.28)	(10)[b]
without nephropathy	130			1.13 (0.11)	(2)[a]
with nephropathy	269	(53)	(7)[a]	1.71 (0.34)	(8)[b]

Mean (SE), or number.
[a] $p < 0.025$ and [b] $p < 0.001$ compared to control subjects.
[c] $n < 0.025$.

diabetes and the difference between the patients with and without retinopathy was not statistically significant (Tables 4 and 5).

Discussion

In various renal diseases including diabetic nephropathy, a number of morphological changes are observed not only in cellular components but also in the extracellular matrix.[10] Diabetic nephropathy is characterized by thickening of capillary basement membranes. Current evidence suggests that an increase in basement membrane synthesis coupled with a normal or decreased rate of degradation leads to an accumulation and thickening of basement membrane.[11–14] Furthermore serum levels of 7S collagen and laminin P1 in patients with diabetes were found to be greater than those in controls.[2] A significant difference in 7S collagen concentration between diabetic patients with signs of microvascular damage and those without microangiopathy has been identified.[2] Earlier studies reported that a radioimmunoassay for specific 7S domains of type IV collagen detected a high molecular weight antigen which was probably intact type IV collagen and small oligomeric variants.[14] In contrast, assay with IV-EIA detected a single peak of high molecular weight antigen which was probably intact type IV collagen.[5,6,9] These results may indicate that measurement of type IV collagen with IV-EIA does not reflect degradation of pre-existing type IV collagen network but *de novo* synthesis.[5,6]

In the present study, serum and urinary concentrations of type IV collagen were significantly elevated in diabetic patients with proteinuria or elevated creatinine. In diabetic patients with chronic renal failure, serum and urinary concentrations of laminin were significantly elevated. These results suggest that serum and urinary concentrations of type IV collagen and laminin are increased in patients with advanced diabetic nephropathy, and suggest that alteration of basement membrane metabolism occurs in patients with diabetes. Urinary concentration of type IV collagen could however reflect both basement membrane metabolism and a disorder of the glomerular filtration barrier.

Extensive studies have been performed to identify a reliable indicator of early glomerular damage in diabetic nephropathy. As yet only microalbuminuria is an established marker to detect early nephropathy.[15,16] Because the urinary concentrations of type IV collagen in diabetic patients with microalbuminuria were significantly higher than in those without nephropathy, the measurement of type IV collagen measured by IV-EIA in urine may be a useful, non-invasive method for the study of the dynamics of glomerular basement membrane and may be a marker for incipient renal damage. Högemann *et al.* reported that serum concentrations of laminin in patients with diabetic microvascular lesions were not significantly different from patients without clinical signs of microangiopathy.[2] Pietschmann *et al.* reported that serum

concentrations of laminin were increased in advanced diabetic nephropathy.[9] We obtained results similar to those of Pietchmann *et al.* However, urinary concentrations of laminin were significantly higher in patients with diabetes, even in those without nephropathy, than in normal controls. These increases may indicate changes of laminin metabolism in glomerular basement membrane.

In less advanced stages of diabetic nephropathy, an increase of type IV collagen and laminin were reported in human renal specimens by immunohistochemical methods.[17,18] We presume that our data of type IV collagen and laminin in patients with diabetes is related to these immunohistochemical results.

Thus this non-invasive measurement of type IV collagen and laminin in serum and urine might be useful in the detection of alterations of the glomerular basement membrane. Furthermore urinary concentrations of type IV collagen might be a useful marker for incipient renal damage.

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